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Molecular and physiological insights into the potential efficacy of CO_2-augmented postharvest cold treatments for false codling moth

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ABSTRACT

Cold sterilisation may be supplemented with elevated CO_2 (hypercapnia) to increase pest mortality however, in some cases, such treatment combinations have generated unexpected high levels of pest survival. Consequently, determining the mechanistic basis of potential interactions or any cross resistance between CO_2 and low temperature stress is critical for effective pest disinfestation through cold sterilization. Here, using larvae of false codling moth *Thaumatotibia leucotreta*, a crop pest in southern Africa, we explored the effects of various hypercapnic pre-treatments on larval survival to standard cold exposures, and a diverse array of biochemical traits that may be indicative of key cellular stress responses or damage and repair processes. Short (< 4 h) pre-treatments with a single stressor (cold or hypercapnia), multiple stress combinations (hypercapnia with hypoxia, and cold hypercapnia), followed by 10 h cold exposure had little effect on larval survival (> 96%). Longer 24 h pre-treatments (hypercapnia + cold) followed by 5 d cold exposure led to significant differences in larval survival (0–80%), with the recovery conditions between exposures greatly affecting larval survival. In contrast with the short-term cold exposures, larvae from the long-term experiments had increased haemocyte mortality, protein concentration and heat shock protein 70 levels, while the concentrations of key cryoprotective sugars were decreased. No changes in membrane lipids could be attributed to the presence of CO_2. These molecular correlates can be used as testable hypotheses for future work to further identify the mode of action of CO_2 reducing (or enhancing) cold tolerance in these insects. From an insect physiology standpoint, chronic hypercapnic cold sterilization protocols appear to be a viable post-harvest option for control of *T. leucotreta*.

1. Introduction

Export of commodities is facilitated by successful post-harvest sterilization protocols. As methyl bromide, an ozone-depleting chemical used as a quarantine treatment is phased out, the demand for developing alternative, effective protocols has grown (Fields and White, 2002). Two environmentally-friendly alternative approaches include extreme temperatures and controlled or modified atmospheres, either alone, or in combination. For commodities that cannot withstand heat treatments, and are already stored and transported at low temperatures, using these temperatures in combination with controlled atmosphere to increase pest mortality is an appealing option. Controlled atmospheres usually consist of combinations of low oxygen (hypoxia) and/or high carbon dioxide (hypercapnia) with the balance consisting of nitrogen. Both combinations, and sequential exposures (e.g. CO_2 followed by cold) can be used successfully to target different pest-produce combinations (Alonso et al., 2005; Palou et al., 2008; Potter et al., 1994; Riudavets et al., 2016; Whiting and Hoy, 1997). The addition of CO_2 above normal atmospheric levels (~0.04%) or the artificial reduction of normal atmospheric oxygen levels (~21.0%) can significantly increase insect mortality under cold conditions. For example, controlled atmosphere cold storage (2.5% O_2, 1–1.5% CO_2, 0 °C) increased larval mortality of codling moth (*Cydia pomonella*) from 4.2% (standard cold storage) to 26% (Moffitt and Albano, 1972). However, in certain cases, combination protocols can improve survival (Mitcham et al., 1997; Soderstrom et al., 1991) suggesting that cross-resistance or interactions between common stress pathways resulted in the undesired outcomes.

It is currently unknown whether increased pest mortality can be attributed to the increased CO_2, or decreased O_2, of combination treatments. In the case of the cowpea bruchids (*Callosobruchus maculatus*) the decreased O_2 is likely responsible for the increased mortality (Cheng et al., 2012). However, for most pests targeted in controlled atmosphere protocols, physiological responses to these gas and temperature stressors have neither been studied individually, nor in

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combinations. Thus, the scope for cross tolerance needs to be investigated, together with the potential time-courses of responses, as acute exposures may elicit cross tolerance, while chronic ones are detrimental and cause insect mortality.

Insect biochemical responses to survive low temperatures typically include the upregulation of various stress-response proteins, alterations to maintain membrane fluidity, increased production of cryoprotective sugars, and maintenance of ion homeostasis (Lee, 2010). The effects of CO_2 on insects have long been studied (Nicolas and Sillans, 1989), especially the effects of CO_2 on ventilation (e.g. Matthews and White, 2011). However, it remains unclear how hypercapnia may interact with standard low temperature stress responses to increase insect mortality in controlled atmosphere protocols (Boardman et al., 2011; Mitcham et al., 2006). Hypercapnia may decrease pH (Stone and Koopowitz, 1974) which would rapidly affect cellular, membrane and protein (including enzyme) functioning necessary for surviving cold exposure. Similarly, nicotinamide adenine dinucleotide phosphate (NADPH) producing enzymes involved in lipid synthesis, cholesterol synthesis and fatty acid chain elongation are decreased by hypercapnia (Friedlander et al., 1984). Hypercapnia also has an anaesthetic effect on insects – through direct stoppage of the heart and blocked synaptic transmission (Badre et al., 2005). One hour of CO_2 blocks rapid cold hardening (a beneficial physiological response to withstand cold) in *Drosophila melanogaster*, while shorter time periods did not elicit this response (Nilson et al., 2006). This highlights the complex interactions of CO_2 exposure time and low temperature responses.

False codling moth *Thaumatotibia leucotreta* (Lepidoptera: Tortricidae) (Meyrick) is a pest of economic importance occurring in southern Africa. The chill-susceptible larvae develop within ripening fruits, and the fifth instars are the most cold tolerant (Myburgh, 1965), and thus cold sterilization protocols are based on the tolerance of this lifestage. Previous work on these larvae by our group has explored the cold tolerance of these pests (Boardman et al., 2012) and shown that physiological responses to hypoxia may interact synergistically with cold responses at shorter timescales (Boardman et al., 2015). This is not unusual as many insects have extreme hypoxia tolerance (Harrison et al., 2006; Hoback and Stanley, 2001) and *T. leucotreta* larvae can survive 36 h of anoxia (Boardman et al., 2016a). Thus, increased carbon dioxide – rather than decreased oxygen – may be a more viable alternative for controlled atmosphere-low temperature post-harvest sterilization protocols for these and other hypoxia-tolerant pests.

Here, we investigated the effects of hypercapnic pre-treatments followed by a standard laboratory cold exposure on the physiology and biochemistry of final instar larvae of *T. leucotreta* in order to better understand the effects of single and combined stressors of post-harvest protocols. We used four sets of CO_2 pre-treatments totalling 13 experimental regimes (Fig. 1) and investigated larval survival, pupation and emergence; as well as haemocyte viability, membrane phospholipid properties, total protein concentration, heat shock protein 70 and five potential cryoprotectants at the end of these experiments to gain insight into the potential mechanisms influencing survival of these stressors. The experiments included various combinations of hypercapnia and cold followed by a standard cold exposure of either 10 h (= short) or 5 d (= long). In addition, the effects of variable recovery periods between the pre-treatment and standard long cold exposure were investigated as it is well known that cold stress responses may require a brief period at a warmer temperature to allow gene expression and protein production (Clark and Worland, 2008). We hypothesized that prolonged hypercapnia would decrease larval survival, likely correlated with an increase in haemocyte mortality and decrease in membrane fluidity (measured as the ratio of unsaturated to saturated fatty acids). Similarly, we expected that reducing the recovery time between pre-treatment and standard cold exposure would increase larval mortality.

2. Materials and methods

2.1. Insects

False codling moth *T. leucotreta* (Meyrick) (Lepidoptera, Tortricidae) larvae were obtained from Cedar Biocontrol Insectary, XSIT (Pty) Ltd, Citrusdal, South Africa and kept in the lab at 25 °C (L:D 12:12 h; YIH DER growth chamber, model LE-539, SCILAB instrument CO Ltd., Taiwan) (Boardman et al., 2012). Larvae were used for experiments when they reached their final instar and wandered off food. Larvae were placed in individual ventilated 1.5 mL tubes and fasted for 24 h prior to the start of the experiments. Sampling for assays was done at the end of each experiment after the standard recovery period.

2.2. Experimental blocks

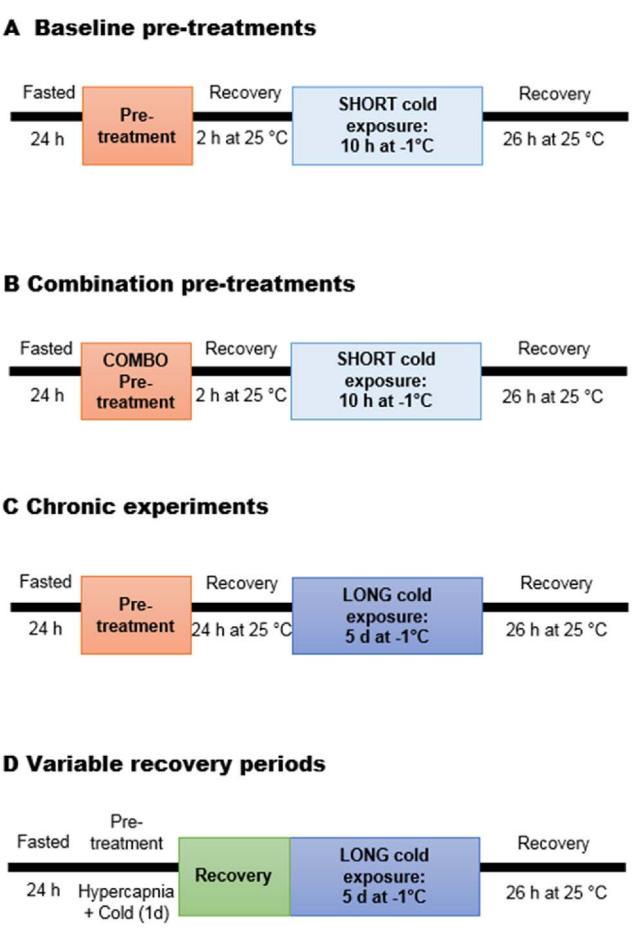
Larvae were exposed to a pre-treatment (details below in 2.2.1 and 2.2.2), followed by a recovery period at 25 °C and a standard −1 °C cold exposure and recovery at 25 °C for 26 h. Four blocks of experiments were used (Fig. 1) to investigate physiological aspects of CO_2 pre-treatments and cold exposures. The first two blocks consisted of either baseline pre-treatments or combination pre-treatments (see 2.2.1) followed by a short 10 h cold exposure. The subsequent blocks consisted of longer pre-treatments and variable recovery periods (see 2.2.2) before a 5 d long cold exposure.

Gas pre-treatments were performed using bottled gas fed directly from a custom mixed K-cylinder (Air Products, South Africa) directed into a plastic container (5.5 L). All gas mixtures were balanced with pure nitrogen. The container was plumbed to allow gas to flow through the container to keep the container under positive pressure and prevent atmospheric air from entering the container. Gas was monitored using a microtube plumbed through the container using an O_2/CO_2 gas analyser (PDA 400, Pacific CA Systems, Yakima, WA). Gases were always dry (< 10% RH), i.e. not humidified.

A fridge was used for 24 h pre-treatments at 4 °C, while chronic experiments were performed on an ice slurry as there was no way to perform the shaking component inside the fridge due to space constraints. The heat pre-treatment was done in a waterbath (Grant TC-150-R4, Grant Instruments, Cambridge, UK) set to 36.5 °C. Standard cold exposures were always −1 °C (± 0.2 °C), obtained using programmable circulating and refrigeration bath (CC410wl, Huber, Germany). Temperature and relative humidity (RH) were monitored using Thermocron iButtons (DS1922L and DS1923, accuracy ± 0.5 °C and 5% RH, Dallas Semiconductors, Dallas, TX, USA).

2.2.1. Short cold exposure experiments

The short cold exposure experiments consisted of a pre-treatment followed by 2 h recovery at 25 °C, a standard 10 h cold exposure at −1 °C, and 26 h of recovery at 25 °C. The baseline pre-treatments block (Fig. 1A) of experiments were chosen to give initial estimates of responses to short-term stressors. The four baseline pre-treatments were: 2 h at 25 °C (handling control); 2 h at 0 °C (cold); 6% CO_2 for 4 h at 25 °C (hypercapnia) and 6% CO_2 with 2% O_2 for 4 h at 25 °C (hypercapnia + hypoxia) (Fig. 1A). The combination of hypercapnia and hypoxia was included as this is a commonly-used controlled atmosphere treatment, and could indicate which gas was responsible for physiological responses. The data for handling control and cold pre-treatments are taken from Boardman et al. (2015). The combinations of CO_2 and cold pre-treatments, as well as the ordering of these stressors were investigated in the combination pre-treatment block (Fig. 1B) using three different pre-treatments: 2 h at 0 °C followed immediately by 6% CO_2 for 4 h at 25 °C (cold → hypercapnia); 6% CO_2 for 4 h at 25 °C followed immediately by 2 h at 0 °C (hypercapnia → cold); and 6% CO_2 for 4 h at 0 °C (hypercapnia + cold) (Fig. 1B).



Pre-treatment	Details
Handling control	2 h at 25 °C
Cold	2 h at 0 °C (>50 % RH)
Hypercapnia	6 % CO_2 for 4 h at 25 °C (<10 % RH)
Hypercapnia + Hypoxia	6 % CO_2 and 2 % O_2 for 4 h at 25 °C (<10 % RH)

Pre-treatment	Details
Cold → Hypercapnia	2 h at 0 °C (>50 % RH) followed immediately by 6 % CO_2 for 4 h at 25 °C (<10 % RH)
Hypercapnia → Cold	6 % CO_2 for 4 h at 25 °C (<10 % RH) followed immediately by 2 h at 0 °C (>50 % RH)
Hypercapnia + Cold	6 % CO_2 for 4 h at 0 °C (<10 % RH)

Pre-treatment	Details
Long hypercapnia + Cold	6 % CO_2 for 24 h at 4 °C (<10 % RH)
Long hypercapnia + Cold (Shake)	6 % CO_2 for 24 h at 4 °C (<10 % RH) with mechanical vibrations
Heat	4 h at 35 °C (>50 % RH)

Recovery	Details
No recovery	Immediate transfer from pre-treatment to cold exposure
Long recovery	3 d at 25°C
Cold recovery	2 h at 25°C, 3 d at -1°C, 2 h at 25°C

Fig. 1. A graphical representation of the experimental design. Baseline pre-treatments (A) and combination pre-treatments (B) were followed by a ten-hour standard short cold exposure. The chronic experiments (C) and variable recovery periods after long hypercapnia + cold (D) experiments were followed by a five-day standard long cold exposure. Full methodological details are provided in text.

2.2.2. Long cold exposure experiments

To make the lab experiments more relevant to cold sterilization protocols, experiments with a longer standard cold exposure were used. These experiments consisted of a pre-treatment followed by a recovery period of 24 h at 25 °C, a standard 5 d cold exposure at −1 °C, and 26 h of recovery at 25 °C. The chronic experiment block (Fig. 1C) used two longer pre-treatments of 24 h of 6% CO_2 at 4 °C (long hypercapnia + cold) or 24 h of 6% CO_2 at 4 °C combined with shaking or vibration stress (long hypercapnia + cold (shake)). The shaking was used to identify whether mechanical stress may also affect stress responses during the long combination pre-treatment. The third experiment in this block was a 4 h heat pre-treatment at 35 °C (heat) which was included as a non-gas stress control for the long cold exposure treatments (Fig. 1C).

The importance of recovery periods was investigated using three experimental regimes (Fig. 1D). A standard pre-treatment of 24 h 6% CO_2 at 4 °C (long hypercapnia + cold) was used, followed by variable recovery periods before the standard 5 d cold exposure and 26 h recovery. The recovery periods were: i) samples moved directly from pre-treatment to standard cold exposure (no recovery); ii) 25 °C for 3 d (long recovery); or iii) 25 °C for 2 h, −1 °C for 3 d, 25 °C for 2 h (cold recovery) (Fig. 1D).

2.3. Assays

All assay methods were performed following Boardman et al. (2015) and are described briefly again below. Experiments were performed with a minimum of n = 100 larvae, and samples for membrane phospholipid composition, proteins and cryoprotective sugar polyols were frozen at −80 °C. Additional live samples for haemocyte viability were obtained by repeating the experiments on a smaller sample size (~n = 10 per experiment). Given the potential difference of acute vs. chronic stress exposures, all data were analysed based on the duration of the standard cold exposure, with comparison made among the seven experiments in the short cold exposure group and the six experiments in the long cold exposure group (Fig. 1). For all traits, a generalised linear model (GLZ) was used to analyse the data in SAS 6.1 (SAS Institute., Cary, NC, USA). Details of the models are given in each subsection. Short and long cold exposure experiments were analysed independently. Significant differences between groups were identified using the *lsmeans* procedure in SAS.

2.3.1. Survival, pupation and emergence

Larvae were assayed for survival at the end of the experiments after the final recovery period at 25 °C. Larvae that were not actively crawling or did not respond to prodding were scored as dead. Those not frozen for other assays were kept at 25 °C (YIH DER growth chamber) to monitor pupation and emergence. Survival, pupation and emergence data were analysed independently with a GLZ with a binomial distribution, logit link function and corrected for overdispersion in SAS 6.1.

2.3.2. Haemocyte viability

In order to determine whether haemocytes were damaged and

Table 1
Summary of results of generalized linear model (GLZ) for the effects of experiment on the variables measured. Significant effects are highlighted in bold font.

Variable	Short cold exposure experiments			Long cold exposure experiments		
	DF	Wald χ^2	*P*-value	DF	Wald χ^2	*P*-value
Survival	7	2277.52	< **0.0001**	6	90.78	< **0.0001**
Pupation	7	100.25	< **0.0001**	6	12.83	**0.046**
Emergence	7	44.42	< **0.0001**	6	83.47	< **0.0001**
Haemocyte viability	7	4409.50	< **0.0001**	6	2364.01	< **0.0001**
UFA:SFA	6	7.62	0.27	5	15.83	**0.007**
FA chain length	6	22.38	**0.001**	5	10.46	0.06
Total protein	6	94.17	< **0.001**	5	5.00	13.52
HSP70	6	49.67	< **0.001**	5	5.00	8.06
Fructose	6	3.55	0.74	5	5.78	0.33
Glucose	6	10.00	0.12	5	3.64	0.60
Maltose[a]	6	27.02	**0.0001**			
Sorbitol[a]	6	6.48	0.37			
Trehalose[a]	6	8.37	0.21			

UFA:SFA – unsaturated fatty acids: saturated fatty acids; FA – fatty acid; HSP70 – heat shock protein 70; DF – degrees of freedom.
[a] Levels were too low to detect in long cold exposure experiments.

whether their damage correlated with overall larval survival rates, haemocyte viability was measured in fresh samples at the end of the experiments, using LIVE/DEAD sperm viability kit (Molecular Probes, Inc., Eugene, OR) (Yi and Lee, 2003). Haemocytes were imaged at 10 X magnification using a live-cell fluorescence Olympus IX 81 microscope with CellˆR software (Olympus Biosystems/Soft Imaging System) and a MT-20 150W xenon illumination source with TxRED and FITC excitation filters. Three different areas of haemocytes were imaged for each larva. Damaged cells fluoresce red or orange-red, while live cells were green in colour. For each image, haemocyte mortality was determined for a minimum of 80 cells using ImageJ software (1.49 v) with grid and cell counter plugins. Data from 3 images were pooled to get 1 value per larva. For short cold exposure experiments (Fig. 1A, B), n = 3 larvae were assayed as survival was high after these experiments. For long cold experiments where some mortality occurred, 2 “normal” and 2 moribund larvae were assayed (n = 4), except for the cold recovery experiment where 3 moribund larvae were assayed as no live larvae were available. The total number of larvae assayed was n = 44. Haemocyte mortality data were analysed with a GLZ with a binomial distribution, logit link function and corrected for overdispersion in SAS 6.1.

2.3.3. Membrane phospholipid composition and cryoprotective sugar polyols

Cryoprotective sugars and polyols were measured using gas chromatographic analysis coupled with mass spectrometry (GC–MS) on a Shimadzu GCMS-QP2010 Plus equipped with an auto-sampler (Columbia, MD, USA) to quantify the changes to membrane phospholipid fatty acid composition and the concentration of five common cryoprotectants (fructose, glucose, maltose, sorbitol and trehalose) in response to the various stressors (Boardman et al., 2015; Waagner et al., 2013). Larvae (n = 4) were freeze-dried and weighed to determine dry mass (accuracy 0.01 mg, Sartorius Ultra Microbalance SC2 (Bradford, MA, USA) before homogenisation in ethanol (sugars) or PO_4 buffer (lipids). As the long cold experiments (Fig. 1C, D) resulted in some mortality, the 4 samples used consisted of 2 live and 2 moribund samples, with the exception of the cold recovery where 4 moribund samples were used as no live larvae were available. Data were not significantly different between live and moribund samples from the same group. The raw data were used to calculate the ratio of unsaturated to saturated phospholipid fatty acids (UFA:SFA) and fatty acid chain length (Waagner et al., 2013) – both indicators of membrane fluidity. Membrane phospholipid composition, fatty acid chain length and cryoprotectant concentrations were analysed independently with a GLZ using a normal distribution and identity link function in SAS 6.1.

2.3.4. Total protein concentration and HSP70

To determine if one of the major stress response proteins, heat shock protein 70 (HSP70), was correlated with the *T. leucotreta* mortality after cold phytosanitary treatments, total protein concentration and HSP70 was determined (full details in Boardman et al., 2013). Larvae from each experiment (n = 3; n = 6 for handling control) were weighed (accuracy ± 0.1 mg; AB104-S/Fact, Mettler Toledo International, Inc.) before being homogenised (total n = 52). The total protein concentration was determined using a Pierce BCA protein assay kit (Thermo-Scientific, full details in Boardman et al., 2013). Thereafter, 30 μg ml^{-1} of total protein was used to measure HSP70 in a standard ELISA procedure (Boardman et al., 2013). ELISAs were performed using monoclonal anti-heat shock protein 70 antibody produced in mouse, clone BRM-22 (1:5000; Sigma H5147) and 1:20000 enzyme-conjugated secondary antibody (HRP-conjugated rabbit anti-mouse IgG, Abcam #6728) in PBS-T. The primary antibody identifies both the constitutive (HSP73) and inducible (HSP72) forms of HSP70, referred to herein as HSP70 for brevity. Total protein concentration and HSP70 expression level were analysed with a GLZ using a normal distribution and identity link function in SAS 6.1.

3. Results

3.1. Short cold exposure experiments

The pre-treatments before the short 10 h cold exposure resulted in significantly different larval survival, pupation and emergence, as well as haemocyte viability, fatty acid chain length, total protein, HSP70 and maltose (all $P < 0.001$, Table 1). The UFA:SFA ratio, and concentrations of fructose, glucose, sorbitol and trehalose were not significantly different between experiments.

Among the baseline pre-treatments (Fig. 1A), cold, hypercapnia and hypercapnia + hypoxia increased survival by ~2% over handling control (Fig. 2). Haemocyte mortality was significantly higher after hypercapnia than handling control and cold pre-treatment levels, and significantly lower after hypercapnia + hypoxia (Fig. 3). Haemocyte mortality was not correlated with whole animal (larval) survival ($y = 81.19 + 0.07x$; $r = 0.349$; $P = 0.24$) as the pre-treatments that had the highest survival did not have the lowest haemocyte mortality. While UFA:SFA was unaffected at the end of the experiments, fatty acid chain length differed, with both cold and hypercapnia + hypoxia increasing fatty acid chain length above handling control levels (Table 1 Fig. 4).

Hypercapnia + hypoxia increased total protein concentration and decreased HSP70 in comparison to the handling control (Fig. 5). While

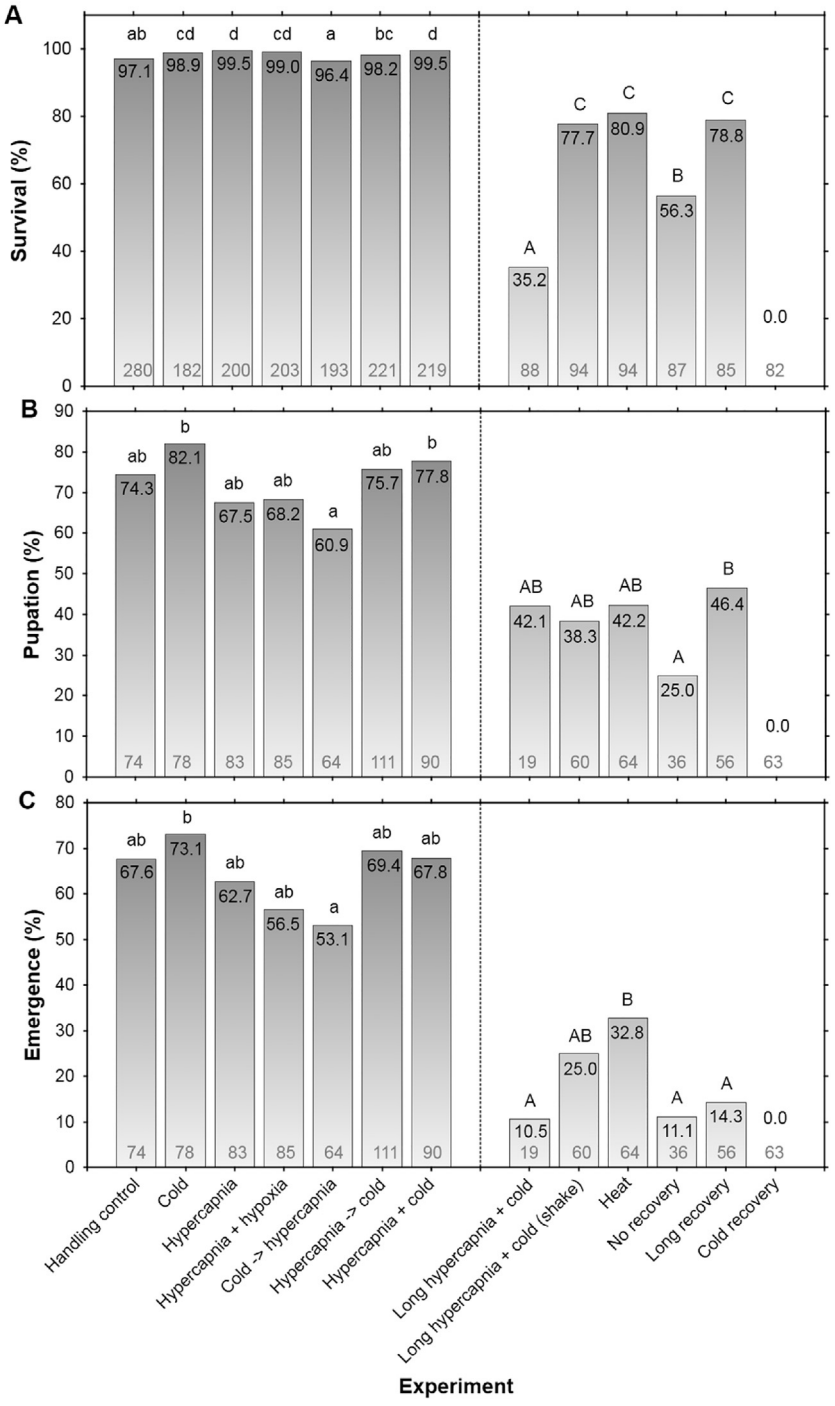


Fig. 2. Survival, pupation and emergence rates of *T. leucotreta*. Significant differences ($P < 0.05$) between experiments are indicated by different letters (small letters for "short cold exposure" [left hand side] experiments and capital letters for "long cold exposure" experiments [right hand side]). Percentage values are shown in black font at the top of the bars as the scaling does not show small but significant changes, while sample sizes are given in grey at the bottom of the bars. Refer to Fig. 1 for experimental treatment details.

total protein concentration was similar between cold, hypercapnia and the handling control, cold pre-treatment significantly increased HSP70 (Fig. 5B). Maltose was the only cryoprotectant that was significantly affected at the end of the experiments. After cold and hypercapnia, the concentration of maltose was significantly less than after the handling control and hypercapnia + hypoxia (Fig. 6C).

The combination pre-treatments were compared to the handling control, as well as the single cold and hypercapnia stress exposures in order to investigate how the hypercapnia and cold combinations affected the larval responses. The order of the combination pre-treatments (Fig. 1B) affected survival and pupation, although all resulted in high survival rates > 96%. Cold → hypercapnia was comparable to the handling control, but resulted in significantly less emergence than cold alone (Fig. 2). The simultaneous stress of hypercapnia + cold increased larval survival and pupation, by almost 3% and 17% respectively, in comparison to cold → hypercapnia (Fig. 2). Hypercapnia → cold resulted in intermediate survival between the other combinations. The combinations of hypercapnia and cold affected haemocyte mortality differently. Hypercapnia + cold resulted in significantly less haemocyte mortality in comparison to hypercapnia and cold sequential pre-treatments (Fig. 3). Cold → hypercapnia and hypercapnia → cold both had higher fatty acid chain length in comparison to handling control, and were similar to cold pre-treatment (Fig. 4). The fatty acid chain length after hypercapnia + cold was also higher than the control, but not significantly so. The order of the hypercapnia and cold combinations had no effect on total protein concentration and HSP70, although all three combination pre-treatments significantly decreased total protein relative to the four baseline pre-treatments, and decreased HSP70

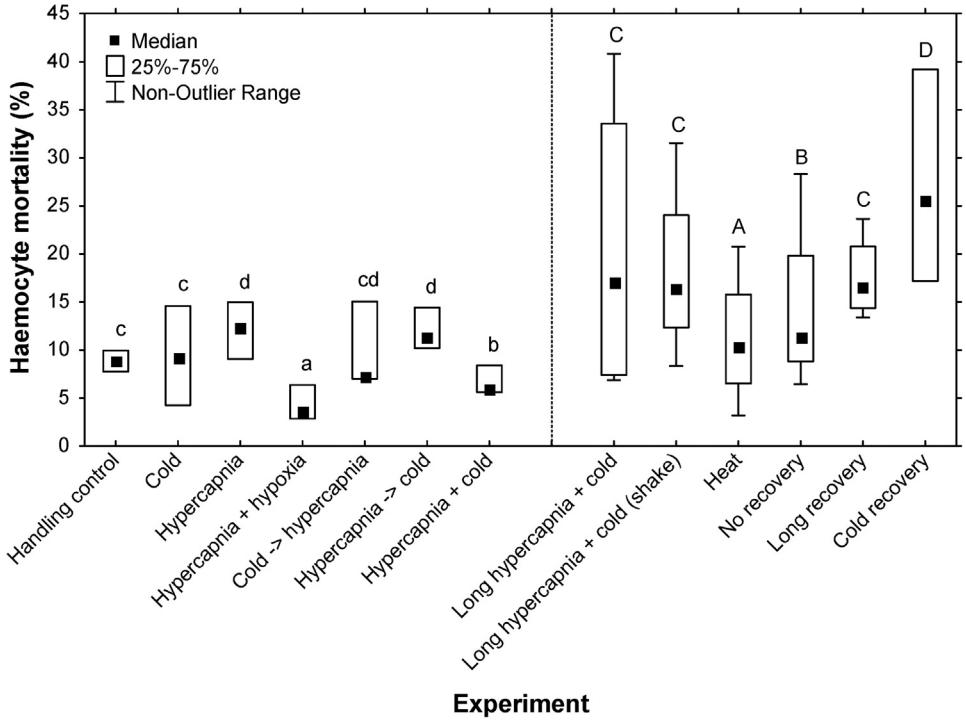


Fig. 3. Haemocyte mortality after each experiment. Significant differences ($P < 0.05$) between experiments are indicated by different letters (small letters for "short cold exposure" experiments and capital letters for "long cold exposure" experiments).

significantly in comparison to the cold pre-treatment (Fig. 5). All three combination pre-treatments decreased the maltose concentration to trace levels that were comparable to both individual stress cold and hypercapnia pre-treatments (Fig. 6C).

3.2. Long cold exposure experiments

The pre-treatments before the standard long cold exposure significantly affected survival, pupation and emergence, haemocyte viability and UFA:SFA ratio (Table 1). Fatty acid chain length, total protein, HSP70, fructose and glucose were not different at the end of the different experimental regimes. Maltose, sorbitol and trehalose could not be detected after all the experiments which hampered statistical comparisons.

The chronic experiments were compared to one another to identify whether mechanical stress acted synergistically or antagonistically with long 24 h hypercapnia + cold. The long hypercapnia + cold, both with and without shaking, were compared to the shorter more acute pre-treatment of 4 h hypercapnia + cold to compare the responses of acute vs. chronic exposures. The mechanical stress of shaking did not affect haemocyte mortality, which was similar after long hypercapnia + cold regardless of whether the shaking was included. Both these experiments had significantly more haemocyte mortality in comparison to heat pre-treatment (Fig. 3). The increase in the duration of the pre-treatment (4 h to 24 h) and standard cold exposure (10 h to 5 d) significantly increased haemocyte mortality (comparison of hypercapnia + cold to long hypercapnia + cold; Fig. 3). Shaking resulted in significantly higher UFA:SFA ratio (Fig. 4). The fatty acid chain lengths, total protein concentration and HSP70 were comparable between the three chronic pre-treatments (Figs. 4 and 5), and none of the cryoprotectants were different after any of the chronic pre-treatments. Maltose, trehalose, and in some experiments, sorbitol, were reduced to trace amounts (Fig. 6).

Lastly, to investigate the role of recovery periods in these experimental protocols, we compared no recovery, long recovery and cold recovery to the hypercapnia + cold pre-treatment from the chronic experiments. The different recovery durations significantly affected haemocyte mortality (Fig. 3). The no recovery experiment decreased haemocyte mortality in comparison to the long hypercapnia + cold experiment that had 24 h recovery at 25 °C. The extended recovery (an additional 2 d at 25 °C) had no effect, while the cold recovery resulted in significantly more haemocyte mortality (Fig. 3) and effectively an 8 d cold treatment. The recovery period did not affect the UFA:SFA ratio, or fatty acid chain length which were all comparable to long hypercapnia + cold. The cold recovery experiment significantly increased total protein concentration relative to no recovery and long recovery, however, HSP70 was not significantly different between these experiments (Fig. 5). Recovery conditions did not affect cryoprotective sugar concentrations (Fig. 6).

4. Discussion

Controlled atmospheres can be used to increase pest mortality rates during post-harvest cold sterilization protocols. Here, our data suggest that elevated carbon dioxide levels in sequential combination with cold stress may be a viable option for the development of more effective post-harvest control measures against *T. leucotreta*. The United States Department of Agriculture requires cold sterilization of consignments of South African citrus, grapes and stone fruit at temperatures at or below −0.55 °C for 22–24 d in order to control *T. leucotreta* (schedule T107-e, T107-k, United States Department of Agriculture, 2016). It is considered that this treatment will also eliminate the fruit flies *Ceratitis capitata*, *C. rosa*, *C. quinaria* and *Bactrocera dorsalis*. It would therefore be necessary to evaluate sequential combinations of carbon dioxide and cold that are effective against *T. leucotreta* against these fruit flies to confirm that they are equally effective before the current cold sterilization could be modified. Previous work has shown that hypoxia may elicit cross tolerance with cold in *T. leucotreta* larvae (Boardman et al., 2015), and that these larvae have some scope for anaerobic metabolism (Boardman et al., 2016a,b). From a commercial viewpoint, it is easier to increase carbon dioxide levels in a hermetic room before fruit is packed and cold treated, than to lower oxygen levels to < 1%, and combinations of CO_2 and O_2 are usually more effective when CO_2 concentrations are high (reviewed in Mitcham et al., 2006).

The baseline pre-treatments indicated that acute exposures to hypercapnia and cold as individual stressors resulted in increased larval

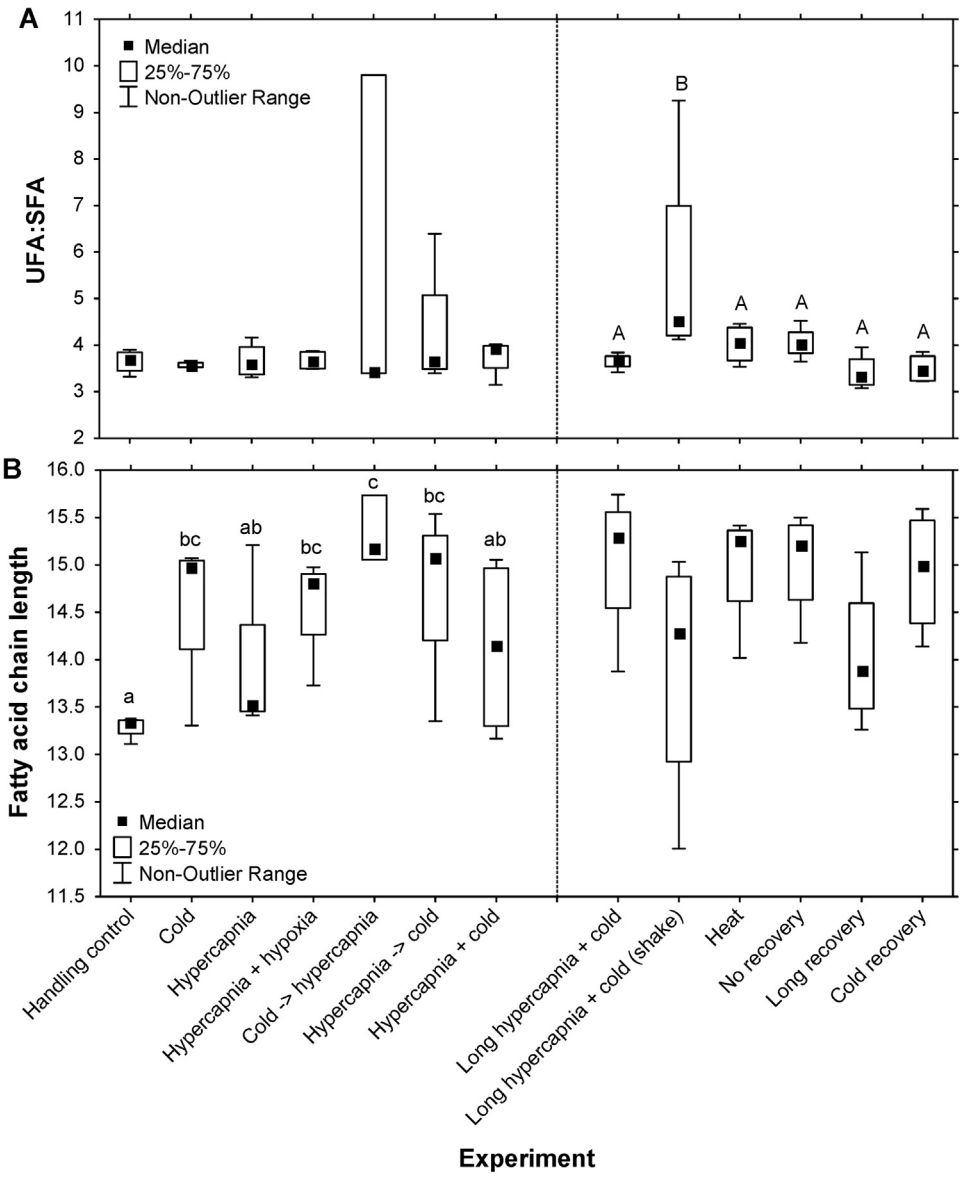


Fig. 4. The ratio of unsaturated to saturated phospholipid fatty acids (UFA:SFA) (A) and fatty acid chain length (B) after each of the experiments. Significant differences ($P < 0.05$) among experiments are indicated by different letters (small letters for "short cold exposure" experiments and capital letters for "long cold exposure" experiments). No significant differences were found between fatty acid chain lengths for long cold exposure experiments.

survival, although pupation and emergence were similar. The 10 h cold exposure to −1 °C was apparently stressful enough to limit emergence to only 67.6%, indicating that some long-term physiological or biochemical damage may have taken place. The combination of hypercapnia and hypoxia was not significantly different to hypercapnia alone in terms of survival, although hypercapnia (without hypoxia) resulted in greater haemocyte mortality, higher HSP70 and significantly less maltose concentration. This suggests that hypercapnia is stressful, resulting in more haemocyte damage and increased HSP70 in comparison to hypercapnia + hypoxia. This may be due, at least in part, to the anoxic nature of the hypercapnia experiment. The decrease in maltose likely indicates an increase in hydrolysis of maltose into glucose to be used as metabolic fuel, perhaps indicating that it does not act as a cryoprotectant in these larvae. Hypoxia appears to reduce the potentially detrimental responses of larvae to CO_2, possibly as a result of the reduction in metabolic rate caused by hypoxia. The cold pre-treatment was the only pre-treatment to increase HSP70 above handling control levels, indicating that HSP70 was not a general stress response in *T. leucotreta*, and was only increased by the cold exposures (also see Boardman et al., 2015a, 2013).

The exact temporal sequence of hypercapnia and cold exposures affected larval survival and pupation rates, but not emergence rates, with hypercapnia + cold resulting in the highest larval survival of any of the experiments (99.5%). While cold → hypercapnia had the lowest survival, it was still high at 96.4%, indicating that none of these acute exposures resulted in significant larval mortality. Both successive stress experiments (cold → hypercapnia and hypercapnia → cold) resulted in an increase in fatty acid chain length (Fig. 4), which could have contributed to the high survival. It appears that the increase in fatty acid chain length may be driven by the cold and hypoxia exposures, more so than by the hypercapnia (Fig. 4). All three combination pre-treatments reduced total protein relative to the other four short cold exposure pre-treatments (Fig. 5). The patterns observed in the total protein reduction following certain cold treatments could be due to changes in the wet mass of the insects, although these masses were not significantly different between handling control and combination pre-treatments ($H_{3,15} = 7.64$, $P = 0.054$). Alternatively, protein production may be reduced by the combination pre-treatments, while protein degradation or regeneration rates continue at the same rates, thus the latter exceed new protein synthesis rates under these conditions. This is largely to be expected from simple theoretical temperature effects on biochemical reaction rates (Boltzmann Arrhenius theory) although further experimentation would be necessary to explore these possibilities. The low HSP70 after the combination pre-treatments may indicate that HSP70 is

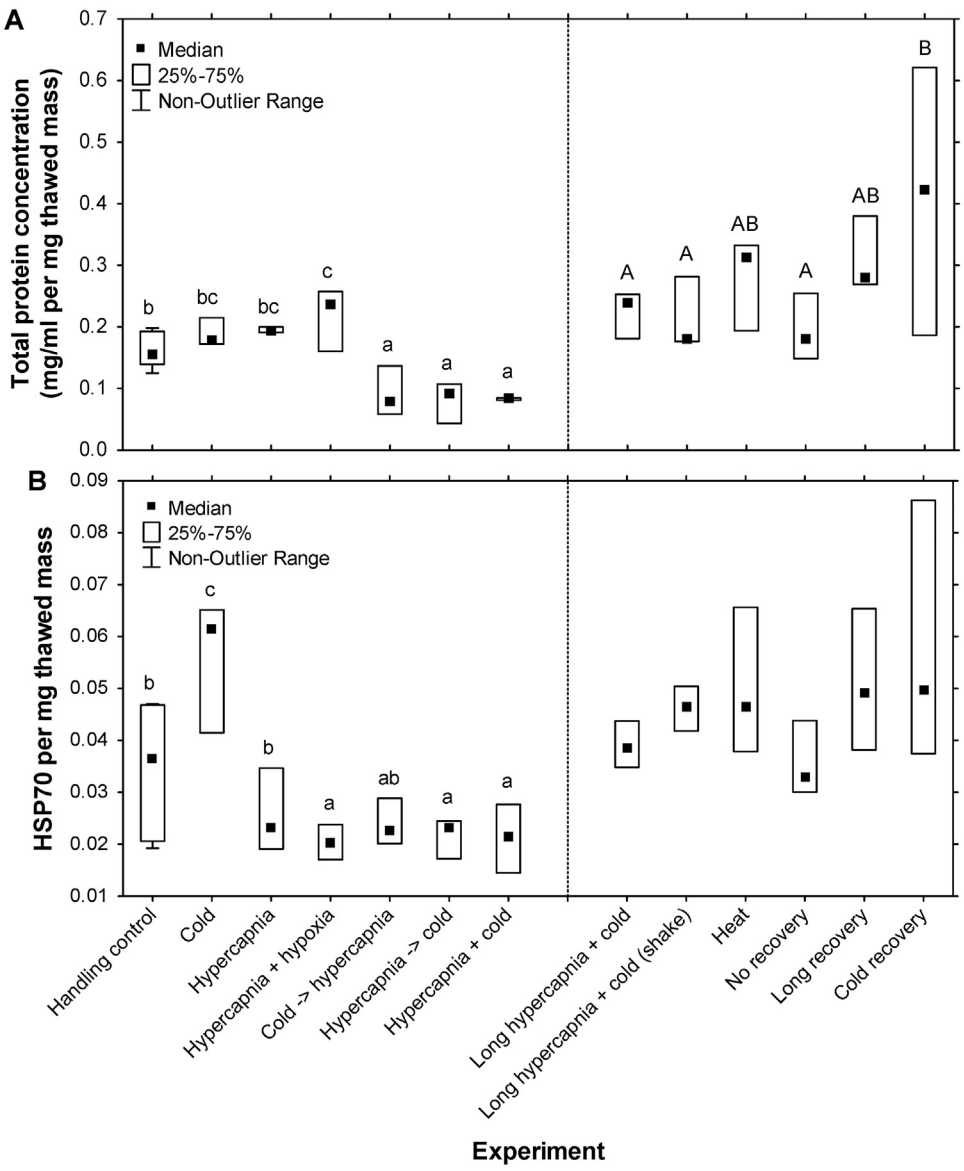


Fig. 5. The total protein concentration (A) and HSP70 expression level (B) after each of the experiments. Significant differences ($P < 0.05$) among experiments are indicated by different letters (small letters for "short cold exposure" experiments and capital letters for "long cold exposure" experiments). No significant differences were found between HSP70 for long cold exposure experiments.

being utilised in response to the stressors or as a moderator of autophagy or apoptosis. Similar to the cold and hypercapnia individual pre-treatments, maltose concentration was reduced to trace amounts after all three combination hypercapnia and cold experiments, possibly indicating its role in providing energy to respond to the stress exposures.

When the standard cold exposure was lengthened to 5 d, in some cases with longer pre-treatments, greater effects on survival were observed. Long-term (24 h) hypercapnia + cold resulted in the lowest survival and lowest emergence rates of treatments that failed to elicit 100% mortality. When shaking was added to this pre-treatment (long hypercapnia + cold (shake)), larval survival was significantly increased, potentially as a result of the increase in UFA:SFA ratio initiated by the shaking (Fig. 4). This did not translate into a long-term benefit though as the individuals that pupated had emergence rates comparable to stress without shaking. The heat pre-treatment before the long cold exposure served as a stress control, as no cross tolerance between heat and cold has been documented (Boardman et al., 2015). This pre-treatment had the highest survival and highest emergence of the chronic pre-treatments prior to the long cold exposure (Fig. 2). The results of the traits studied suggest that this pre-treatment was likely less stressful for the larvae than the long hypercapnic pre-treatments. Mortality resulting from this exposure was likely a result of accumulative cold injury from 5 d spent at −1 °C and not the short heat exposure (Terblanche et al., in preparation). All three of these chronic experiments resulted in an increase in HSP70 compared to the acute hypercapnia + cold experiment, resulting from the additional time spent exposed to −1 °C (5 d vs 10 h).

The recovery conditions between the long (24 h) hypercapnia + cold pre-treatment and long cold exposure (5 d) played a key role in determining survival. No recovery period increased larval survival by 20.9% in comparison to 24 h recovery, however, emergence rates remained unchanged. This suggests that some damage in those larvae that survived initially had probably manifested later during development. When the larvae had 3 d of recovery (long recovery), survival was 22% higher than after no recovery period, although emergence rates remained low. An additional 3 d of cold stress during recovery (cold recovery) killed all the larvae. Although the mechanism for this pronounced effect was not clear from our data, the increase in haemocyte mortality and total protein indicate large stress responses. All measured sugars had low concentrations at the end of this experiment, potentially indicating insufficient metabolic reserves to counteract this additional stress. Carbon dioxide can increase glycogen consumption (Friedlander and Navarro, 1979). It is possible that this increase is what caused the utilization of sugars measured in our data. In other studies, maltose and

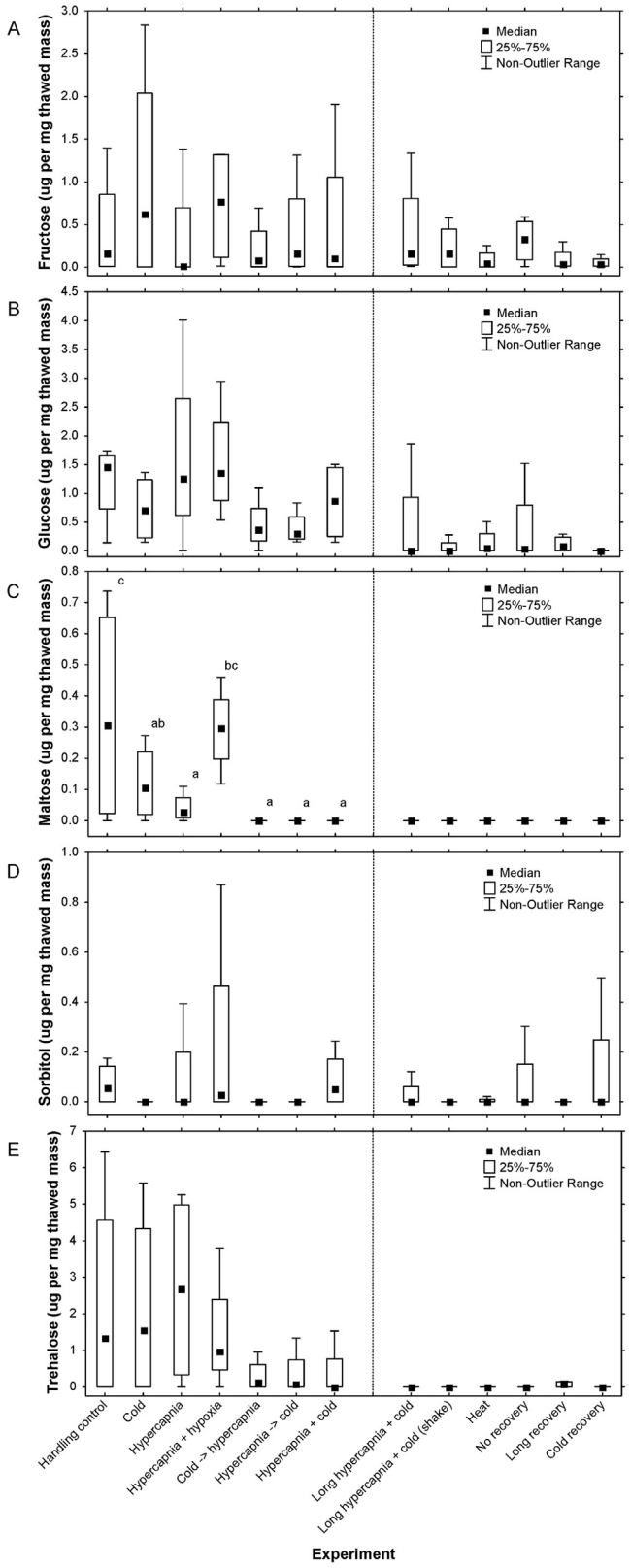


Fig. 6. Cryoprotectant concentrations of fructose (A), glucose (B), maltose (C), sorbitol (D) and trehalose (E) after each of the experiments. Significant differences ($P < 0.05$) between experiments are indicated by different letters (small letters for "short cold exposure" experiments, no differences in "long cold exposure" experiments).

glucose increase in response to cold exposure (Overgaard et al., 2007) and are thought to help stabilise biological membranes. If these resources are depleted after exposure to hypercapnia, and unavailable in response to cold, this may contribute to increased larval mortality.

Another alternative hypothesis that warrants further investigation is the role of glutathione and other anti-oxidants in surviving hypercapnic exposures. Anti-oxidants are frequently implicated in thermal stress responses (Joanisse and Storey, 1996; Lalouette et al., 2011) and glutathione has been shown to increase in response to hypoxia and cold in *T. leucotreta* larvae (Boardman et al., 2016b). However, CO_2 exposure decreases glutathione biosynthesis in *Ephestia cautella* pupae (Friedlander and Navarro, 1984). Should this occur in Lepidopteran larvae too, the larvae will be left without the antioxidant properties of glutathione during the subsequent cold exposure (Joanisse and Storey, 1996), perhaps exacerbating any impacts.

Our results suggest that post-harvest protocols involving combined stressors of CO_2 and cold could be a viable post-harvest disinfestation method for *T. leucotreta*. The data show that even fairly low – by industry standards – levels of hypercapnia (6%) can increase larval mortality after cold treatment and, in effect, the hypercapnia serves as an alternative to an extended cold treatment. Thus, there may be scope to reduce the amount of CO_2 used, or the duration of the cold exposure (also see Moore et al., 2017), in some pest-produce combinations that may be affected by high CO_2. While our data cannot discern whether the effects seen result from the CO_2 itself, or a lack of O_2, our research has shown that these larvae readily survive 24 h of anoxia (Boardman et al., 2016a). Furthermore, larvae are capable of anaerobic metabolism, so the lack of aerobic metabolism can be tolerated over shorter timescales. We also have not yet investigated how the CO_2 used in our study affects the pH of the larval haemolymph. Insect haemolymph is able to hold more CO_2 at lower temperatures (16 °C vs 38 °C), and is well-adapted to functioning at high CO_2 partial pressure (Levenbook, 1950). However, if the buffer capacity of haemolymph was overwhelmed, the decrease in pH would affect enzyme functioning − which is key for survival of cold exposures.

One final implication of this work is that we found evidence that mechanical stress, e.g. shaking or machine vibrations, should preferably be avoided during post-harvest protocols as this can reduce the desired mortality rate of hypercapnia + cold exposure. Although there was little improvement in emergence rates, larval mortality is preferable to delayed mortality because live larvae in commodities are not tolerated at ports of entry. Similarly, chronic cold exposures of only 5 d resulted in low emergence rates, regardless of pre-treatment, showing that damage had occurred that inhibited normal development. While delayed cold-induced mortality may still be sufficient for achieving post-harvest sterilization success, augmentation with sequential CO_2 fumigation may decrease larval survival, likely better satisfying overseas markets. Future research needs to investigate these responses of insects exposed to potential protocols while in fruit. As fruit can buffer the insects from direct exposure, it remains unknown whether longer treatments and/or more severe conditions would be needed to ensure pest mortality. In conclusion, this work has shown mechanisms upon which gas and cold stressors might interact, while supporting the design of potential new post-harvest disinfestation treatments for this and other pest Lepidoptera species using combinations of CO_2 and cold.

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